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Neutralization Epitopes of the F Glycoprotein of Respiratory Syncytial Virus: Effect of Mutation upon Fusion Function

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Eighteen neutralizing monoclonal antibodies (MAbs) specific for the fusion glycoprotein of the A2 strain of respiratory syncytial virus (RSV) were used to construct a detailed topological and operational map of epitopes involved in neutralization and fusion. Competitive binding assays identified three nonoverlapping antigenic sites (A, B, and C) and one bridge site (AB). Thirteen MAb-resistant mutants (MARMs) were selected, and the neutralization patterns of the MAbs with either MARMs or RSV clinical strains identified a minimum of 16 epitopes. MARMs selected with antibodies to six of the site A and AB epitopes displayed a small-plaque phenotype, which is consistent with an alteration in a biologically active region of the F molecule. Analysis of MARMs also indicated that these neutralization epitopes occupy topographically distinct but conformationally interdependent regions with unique biological and immunological properties. Antigenic variation in F epitopes was examined by using 23 clinical isolates (18 subgroup A and 5 subgroup B) in cross-neutralization assays with the 18 anti-F MAbs. This analysis identified constant, variable, and hypervariable regions on the molecule and indicated that antigenic variation in the neutralization epitopes of the RSV F glycoprotein is the result of a noncumulative genetic heterogeneity. Of the 16 epitopes, 8 were conserved on all or all but 1 of 23 subgroup A or subgroup B clinical isolates.

Respiratory syncytial virus (RSV) is the most important cause of severe lower-respiratory-tract illness in young children worldwide. This paramyxovirus causes yearly epidemics during which reinfection of seropositive individuals is common. Although RSV is considered to be a monotypic virus, two antigenic subgroups have been characterized by cross-neutralization studies (8, 9, 54) with monoclonal antibodies (MAbs) (4, 32, 36, 46, 49) and polyclonal sera (50). Subgroup-specific antigenic differences occur in several of the RSV proteins, including the envelope glycoproteins, F and G, and the internal proteins N, M, NP, and the 22-kilodalton (M_2) protein (2, 19, 20, 30, 32, 41, 46, 52). It has been suggested that this antigenic variation is a factor in allowing reinfection to occur (8, 30, 37) and may be a challenge to the development of a vaccine that will give protection against disease from infection with all RSV strains.

The prototype subgroup A strains (A2, Long, and RSS-2) and subgroup B strains (18537 and WV4234) not only differ antigenically but also have considerable nucleotide and amino acid sequence divergence between their envelope glycoproteins (5, 13, 24a, 26). Despite these differences, postinfection sera from cotton rats contain antibodies that will neutralize heterologous strains in vitro, and cross-protection against heterologous challenge has been shown following immunization with either the A2 or 18537 strain in vivo (25, 40). This is due, in large part, to the immune response to the F glycoprotein (28, 33, 34), as demonstrated by the protection against heterologous challenge that was given to animals immunized with a recombinant vaccinia virus expressing the F glycoprotein of RSV A2 (Vac-F) and challenged with strain 18537 (35). These results, however, cannot account for the epidemiologic patterns of RSV infection within the human population: subgroup A and B viruses may either cocirculate or independently dominate epidemics (21), and children so exposed appear to have only partial protection against infection, which is greater against viruses of the same subgroup (31). It is likely that a better understanding of the complex epidemiology of RSV will be facilitated by a detailed analysis of the neutralization and fusion domains of the RSV envelope glycoproteins.

We initiated this analysis by isolating and studying a large panel of neutralizing anti-F MAbs to subgroup A strains of RSV in order to map the antigenic sites and neutralization epitopes within these sites and to study the functional organization of the RSV F glycoprotein. Competitive binding assays of 18 MAbs delineated three antigenic sites, and neutralization assays of the MAbs with MAb-resistant mutants (MARMs) and clinical isolates detected 16 epitopes, some of which appear to be operationally interdependent although topographically distinct. Six of the MARMs selected with fusion-inhibiting MAbs displayed a small-plaque phenotype, indicating that certain neutralization epitopes reside within domains which affect the fusion function of the molecule. These particular epitopes were shown to be conserved among all subgroup A and B RSV clinical strains examined. We also sought to obtain an extensive map of the neutralization and fusion epitopes (i.e., epitopes recognized by MAbs which inhibit fusion between infected cells) by using biological assays which could be used to relate biological functions of this protein to specific antigenic sites.

MATERIALS AND METHODS

Cells and virus. Cells used in these studies (HEp-2, Madin-Darby bovine kidney [MDBK], LLC-MK_2, and BS-C-1) were maintained in Eagle modified minimum essential medium supplemented with 10% fetal bovine serum. Semiconfluent cell monolayers were infected with RSV at a multiplicity of infection of 0.1 in Eagle modified minimum essential medium–1% fetal bovine serum. Extracellular virions were harvested after extensive cytopathology was evident (48 to 72 h postinoculation) and stabilized by the

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addition of 100 mM $MgSO_4$ (16). Viruses used for immunization of mice or for radioimmunoassays were purified by sedimentation through 30% sucrose onto a cushion of 60% sucrose. Virus stocks used for the selection of MARMs were concentrated by centrifugation to 10^8 50% tissue culture infectious doses ($TCID_{50}$) per ml.

The prototype human RSV strains (A2, Long, and 18537) were plaque purified three times in HEp-2 cell monolayers. Other human RSV strains, generously supplied by R. M. Hendry, Food and Drug Administration, Washington, D.C., and H. W. Kim, Children's Hospital National Medical Center, Washington, D.C., were not plaque purified and were passaged a limited number of times in HEp-2 cells; they are identified according to geographic location, isolate number, and year of isolation. The bovine RSV strain was a gift from M. H. Smith, University of Iowa, Ames, Iowa, and was propagated and assayed in MDBK cell monolayers.

Vaccinia virus recombinants expressing RSV F (Vac-F), RSV G (Vac-G), RSV N (Vac-N) or *Escherichia coli* β-galactosidase (Vac-β-gal) were a gift from R. A. Olmsted, Georgetown University, Rockville, Md. Vaccinia virus recombinants were propagated in BS-C-1 cell monolayers (35), and extracellular virions were used for mouse immunization and for infection of cell monolayers which served as antigens in an enzyme-linked immunosorbent assay (ELISA).

Production of hybridoma cell lines. Female BALB/c mice were immunized by primary intranasal infection with 10^5 $TCID_{50}$ of the A2 strain of RSV, and 3 weeks later they were inoculated intraperitoneally with 10^8 PFU of the Vac-F recombinant. Four weeks later, they received an intravenous injection with 10^4 $TCID_{50}$ of purified RSV. Four days after the final immunization, splenic lymphocytes were fused with the NS-1 murine myeloma cell line by standard procedures (27). Hybridoma supernatants producing RSV-specific antibodies were identified by an ELISA of RSV-infected cell monolayers. All positive hybridomas were immediately subcloned twice by the limiting-dilution method and were used for the production of ascites fluid.

Seven additional hybridoma cell lines used in this study were kindly provided by L. Anderson, Centers for Disease Control, Atlanta, Ga., E. Norrby, Karolinska Institute, Stockholm, Sweden, and B. Fernie, Georgetown University; Rockville, Md.; the methods used for their production and characterization have been published (MAb 151 [31]; MAbs 43-1 and 13-1 [15]; MAbs 1436C, 1302A, 1308F, and 1331H [3]). The immunoglobulin subclass of each MAb was determined by using the Boehringer-Mannheim Immunoglobulin Subclass Determination Kit. Polyclonal antisera used in these studies included cotton rat post-RSV-infection serum, pooled adult human serum, and murine anti-RSV ascites fluid. Murine ascites fluids were produced by infection with either RSV or Vac-F, followed by intraperitoneal injection of pristane and Sarcoma 180 cells (47).

Serologic assays. An indirect ELISA was performed with RSV-infected $LLC\text{-}MK_2$ cell monolayers or vaccinia virus recombinant-infected BS-C-1 cells. Cell monolayers in 96-well plates were infected at a multiplicity of infection of 1. After development of extensive cytopathology, the cell monolayers were desiccated at 37°C and stored at 4°C. Plates were washed three times with phosphate-buffered saline–0.1% Tween 20, blocked with 20% fetal bovine serum, and washed again prior to addition of the test MAbs. After an overnight incubation, peroxidase-conjugated goat-anti-mouse immunoglobulin G (Cappel Laboratories) was added for 4 h, and the test was developed with a substrate solution containing the chromophore 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS).

Neutralization assays were performed as follows. Serial twofold dilutions of heat-inactivated (56°C for 30 min) ascites fluid of serum were mixed with 100 $TCID_{50}$ of RSV in the presence of 5% guinea pig complement. After 60 min, the virus-antibody mixtures were transferred to monolayers of HEp-2 cells in 96-well plates; 6 days later, the monolayers were examined microscopically for cytopathology. Microscopic observations were confirmed by staining with 5% glutaraldehyde–1% crystal violet. Neutralization titers were expressed as the reciprocal of the highest antibody dilution which inhibited more than 95% of the viral cytopathic effect present in the control wells.

Fusion inhibition (FI) assays were performed as described previously (32) to identify MAbs that inhibit syncytium formation. Briefly, confluent monolayers of HEp-2 cells in 96-well plates were infected with 100 $TCID_{50}$ of virus at 37°C. After 60 min the inoculum was removed, and the monolayers were washed for 2 h with Eagle modified minimum essential medium to remove unabsorbed virus. Serial twofold dilutions of MAbs or hyperimmune sera were then added to each well. Six days later monolayers were examined for cytopathic effect, and antibody titers were determined in the same manner as described for neutralization assays. Competitive solid-phase radioimmunoassays were performed as previously described (12). Briefly, MAbs were intrinsically labeled with [^{35}S]methionine. Unlabeled ammonium sulfate-precipitated MAbs had a final concentration of 1 mg of protein per ml and showed similar ELISA titers (<10-fold difference) when tested on the RSV A2-infected $LLC\text{-}MK_2$ cells prior to use in the RIA (data not shown). Purified RSV virions were adsorbed to polyvinyl chloride plates at a concentration of 0.15 to 1.4 μg of protein per well. A previously determined saturating amount of labeled MAb was mixed with increasing concentrations of unlabeled ammonium sulfate-precipitated MAbs and added to the wells. The amount of bound labeled MAb was measured for each well. Competitive binding titers were expressed as the reciprocal of the dilution of competing unlabeled MAb which produced 50% inhibition in binding of the labeled MAb.

Selection of MARMs. The method for selection of MARMs has been described previously for influenza A virus (18). Briefly, undiluted ascites fluids were incubated with undiluted or decimal dilutions of concentrated of RSV A2 at 37°C for 1 h. Virus-antibody mixtures were adsorbed to HEp-2 cells for 2 h at 37°C and then overlaid with 0.8% agarose L-15 medium containing the selecting MAb at a dilution of 1:100. After 6 days, plaques were visualized with neutral red. The frequency at which MARMs occurred was estimated as described previously (18, 44). MARMs were plaque purified twice, amplified in the presence of the selecting MAb, and tested again in neutralization assays against the complete panel of anti-F MAbs. MARMs are identified by the prefix V and by the MAb used for their selection (e.g., V1269).

RESULTS

Specificity of MAbs for RSV F glycoprotein. To produce a large number of neutralizing MAbs specific for the RSV F glycoprotein, we immunized mice by sequential infection with RSV A2 and Vac-F recombinant virus. This immunization protocol stimulated high levels of circulating antibodies which reacted in the ELISA with F glycoprotein expressed by Vac-F-infected BS-C-1 cells. Following initial infection with RSV, mice developed anti-F titers of ca. 1:640, which

TABLE 1. Assignment of RSV glycoprotein F-specific monoclonal antibodies to antigenic sites by competitive radioimmunoassay

Antigenic site	[^{35}S]methionine-labeled MAb	Reciprocal dilution of indicated unlabeled MAb which produced 50% inhibition of labeled MAb[a]																
		1436C	1153	1142	151	1200	1214	1237	1129	1121	1107	13-1	43-1	1112	1269	1243	1331H	1308F	1302A
A	1436C	64	256	64	256	64	16	64	64	256	64								
	1153	4,096	1,024	256	1,024	256	256	256	1,024	1,024	1,024								
	1142	16,384	4,096	1,024	16,384	1,024	4,096	4,096	4,096	1,024	4,096								
	151	4,096	1,024	256	4,096	256	64	64	256	1,024	1,024								
	1200	256	1,024	256	256	256	256	256	256	256	256								
	1214	1,024	1,024	256	4,096	256	256	256	1,024	1,024	1,024								
	1237	1,024	1,024	1,024	256	1,024	256	256	1,024	1,024	1,024								
	1129	4,096	1,024	256	1,024	1,024	256	1,024	256	4,096	256								
	1121	256	256	256	1,024	256	64	256	256	1,024	256								
AB	1107	4,096	1,024	1,024	256	4,096		64	1,024	1,024	256								
B	13-1										4,096	1,024	1,024	16,384	1,024				
	43-1										4,096	4,096	4,096	4,096	4,096				
	1112										64	256	64	256	64				
	1269										256	256	64	256	256				
C	1243															256	256	1,024	1,024
	1331H															4,096	16,384	65,536	16,384
	1308F															64	256	256	64
	1302A															16,384	16,384	16,384	4,096

[a] Each MAb was radioactively labeled, added to dilutions of unlabeled MAb competitors, and tested in solid-phase radioimmunoassays. Blanks indicate no competition above that observed with control ascites.

increased to a titer greater than 1:10^6 following subsequent infection with the Vac-F recombinant. Fusion of splenic lymphocytes from animals immunized in this way yielded 30 hybridoma cell lines which produced antibodies to RSV proteins (data not shown). Seventeen of the MAbs reacted specifically in the ELISA with Vac-F-infected BS-C-1 cell monolayers. These MAbs did not react with uninfected monolayers or with monolayers infected with Vac-G, Vac-N, or Vac-β-gal and are therefore designated RSV-anti-F MAbs. The specificity of these MAbs for the viral F glycoprotein was confirmed by radioimmunoprecipitation assays (data not shown).

Hybridoma cell lines were grown as ascites tumors, and the neutralizing antibody titer of each was determined with the RSV A2 strain. Of the 17 F-specific MAbs, 11 were able to neutralize virus infectivity. Seven additional neutralizing anti-F MAbs kindly supplied by three other laboratories were also used in these studies to complete the panel of 18 RSV-anti-F neutralizing MAbs.

Topographical analysis of RSV-F antigenic sites. To determine the number of nonoverlapping antigenic sites detectable with the panel of 18 neutralizing MAbs, we performed competitive-binding solid-phase radioimmunoassay. Each of the 18 MAbs was intrinsically labeled with [^{35}S]methionine, and increasing amounts of unlabeled MAb were used for competition. Competition between two MAbs indicated that they bound to epitopes that were in close proximity, i.e., the same site, whereas lack of competition indicated that they bound to spatially separated sites. The level of competition between labeled and unlabeled MAbs is indicated in Table 1. The endpoints for 50% competition by a given MAb varied over a wide range of antibody dilutions (e.g., the endpoint at which MAb 1214 inhibits binding of other site A MAbs ranges from 1/16 to 1/4,096), presumably reflecting differences in antibody affinities and proximity of epitopes. These differences are unlikely to be due to quantitative differences in the unlabeled MAbs, because they had similar ELISA titers when tested individually. As expected, in every case the binding of labeled MAb was inhibited by the homologous competitor. Ten MAbs exhibiting reciprocal competitions were assigned to antigenic site A, four MAbs (13-1, 43-1, 1112, and 1269) were assigned to antigenic site B, and four MAbs (1243, 1308F, 1331H, and 1302A) were assigned to a region of the F glycoprotein designated site C. MAb 1107 competed reciprocally with MAbs from site A and nonreciprocally with each of the MAbs from site B, indicating that MAb 1107 bound to an epitope that may act as a bridge (site AB) between these two sites. Five nonreciprocal reactions are defined by the pairwise competitions between MAb 1107 (site AB) and MAbs 1214 (site A), 13-1, 43-1, 1112, and 1269 (site B). Such results may also be due to subtle differences in antibody affinities. Alternatively, binding of one MAb may cause conformational changes in other epitopes, which prevent antibody binding of another MAb.

Selection of MARMs and construction of an operational epitope map. Analysis of reactivity patterns of the panel of MAbs with MARMs identified 14 epitopes located within antigenic sites A, B, AB, and C. MARMs were selected from a concentrated preparation of plaque-purified RSV (A2) and were shown to be true mutants by their resistance to neutralization by the selecting antibody. Cross-neutralization studies were done by using 13 MARMs and the complete panel of 18 RSV-anti-F MAbs (Table 2). These neutralization reactivity patterns were used to construct an operational map of the F-glycoprotein epitopes.

An interesting feature of this analysis of the F glycoprotein was that MAbs frequently did not react with MARMs selected by using MAbs assigned to different antigenic sites by competitive-binding assays. This phenomenon was observed for MAbs mapping to all of the F antigenic sites and may reflect the sensitivity of neutralizing MAbs to alterations in areas of the protein other than the actual epitope. For example, the nine MAbs defining antigenic site A by competitive-binding assays (Table 1) clearly recognized four epitopes (A1, A2, A4, and A5), as indicated by their reactivity patterns with MARMS selected by using MAbs map-

TABLE 2. Determination of the number of epitopes within antigenic sites by neutralization of MARMs

Antigenic Site	Epitope	MAb	Monoclonal Antibody Resistant Mutants Selected with the Indicated MAb[a]												
			A									AB	B	C	
			V1436C	V1153	V1142	V151	V1200	V1214	V1237	V1129	V1121	V1107	V1269	V1302A	V1308F
A	1	1436C	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒			
		1153	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒			
		1142	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒			
	2	151	▒		▒	▒	▒	▒	▒	▒	▒	▒			
		1200	▒		▒	▒	▒	▒	▒	▒	▒	▒			
		1214	▒		▒	▒	▒	▒	▒	▒	▒	▒			
	3	1237	▒		▒	▒	▒	▒		▒	▒	▒		▒	
	4	1129	▒		▒	▒	▒	▒		▒	▒	▒			
	5	1121	▒		▒	▒	▒		▒	▒	▒	▒			
AB	6	1107	▒		▒	▒	▒	▒	▒		▒	▒			
B	7	13-1[b]	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒		■	
	8	43-1[b]	▒	▒		▒	▒	▒	▒	▒	▒	▒	▒		
	9	1112		▒		▒	▒	▒	▒	▒		▒			
	10	1269											▒		
C	11	1243		▒		▒				▒	▒			■	■
	12	1331H							▒					▒	■
	13	1308F								▒				▒	■
	14	1302A										▒		▒	■
a FREQUENCY[c]			10^{-4}	10^{-6}	10^{-6}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-6}	10^{-6}	10^{-6}	10^{-4}	10^{-4}	10^{-5}
b PLAQUE SIZE[d]			S	S	N	N	S	S	N	N	S	S	N	N	N

[a] Symbols: ▒, neutralization titer ≤ 80; □, neutralization titer > 80.

[b] MAbs 43-1 and 13-1 were produced following immunization of mice with the Long strain of RSV and do not neutralize the A2 strain, which was used as the parent for the MARMs.

[c] The frequency at which MARMS were selected was calculated according to the following formula: PFU in the presence of RS MAb/PFU in the absence of RS MAb.

[d] Plaque size: N, normal plaque size (2.5 mm); S, small plaque size (0.5 mm).

ping topographically to site A. However, the lack of reactivity of MAb 1237 with variant 1302A (selected with a site C MAb) indicated that it recognizes a fifth epitope (A3) and that its neutralizing activity is influenced by alterations in site C. These results support the view that in some cases, topographically distinct antigenic sites are operationally or conformationally interdependent.

In addition to their altered antigenic properties, approximately half of the MARMs analyzed also exhibited an altered plaque morphology (Table 2). Six of the site A and AB MARMs produced pinpoint plaques under agarose overlay, but this phenotype did not strictly correlate with specific site A epitopes. For example, only two of the three MARMs selected with MAbs recognizing epitope A1 exhibited the small-plaque phenotype. Whether these phenotypic changes reflect differences in the nature or location of mutations in the MARM F proteins is currently unknown.

Antigenic site B is a complex site which was clearly distinguished from site A by MAbs 13-1, 43-1, 1112, and 1269 in competitive-binding assays (Table 1). These results were confirmed by the ability of MAb 1269 to neutralize all site A MARMs and its inability to neutralize a site B MARM (V1269). In several cases, site B MAbs did not neutralize site A MARMs. This lack of neutralization can be explained for MAbs 13-1 and 43-1: these MAbs were produced to the Long strain of RSV and do not neutralize either the A2 strain or most of our site A MARMs (which are mutants selected from the A2 strain). The neutralizing activity of MAbs 13-1, 43-1, and 1112 is clearly influenced by mutations within both sites A and B, as evidenced by their reactivity patterns with site A and B MARMs. Therefore, the results of competitive-binding assays (Table 1), in combination with analysis of mutants (Table 2), indicated that MAbs 13-1, 43-1, 1112, and 1269 recognized four epitopes in site B and that mutations in site A can, in some cases, influence the neutralizing activity of antibodies directed to site B epitopes. For example, V1142 has a site A mutation but is now neutralized by MAb 43-1 (neutralization tier 1/2,560).

MAbs 1243, 1308F, 1331H, and 1302A were unable to neutralize MARMs V1302A and V1308F, confirming our assignment of these MAbs to antigenic site C based on competitive-binding data (Table 1). However, as described above for site B MAbs, the site C MAbs showed unique reactivity patterns with MARMs selected with site A MAbs (V1153, V151, V1237, V1129, and V1121), indicating that they recognized four different site C epitopes which are influenced by changes within site A.

The mutation rate in the RSV-F epitopes was estimated from the frequency of isolation of MARMs (Table 2). MARMs were selected at a frequency of 10^{-4} to 10^{-6}, which is similar to results with other RNA viruses (44).

All MARMs were neutralized by hyperimmune antiserum (data not shown), as described for influenza virus (53). It was noted, however, that MARMs with alterations affecting the binding of MAbs in all three antigenic sites were neutralized less efficiently than MARMs with alterations affecting only one site.

Antigenic variation among RSV clinical isolates from 1956 to 1986. In an attempt to identify additional epitopes within the antigenic sites and in order to determine the degree of antigenic variation that occurs naturally among RSV strains, we tested subgroup A and B viruses recovered during the past 29 years in cross-neutralization assays with the panel of anti-F-MAbs and with pooled human adult sera. The neutralization test was used to define this pattern because it is an important functional assay that might have more biological relevance than binding assays have. There were nine reactivity patterns for the 18 subgroup A viruses tested and five

TABLE 3. Antigenic variation in the neutralization epitopes of RSV subgroup A and subgroup B isolates obtained from 1956 to 1985

Antigenic site	Epitope	MAb	Reciprocal of neutralizing titer[a] of the F-specific MAb against the indicated strain of RSV[b]															% of subgroup A or B viruses neutralized[c]	
			Subgroup A									Subgroup B					BRS[d]		
			W/Long/56 (5)	W/11657/60 (2)	Aus/A1/61 (2)	Aus/A2/61 (1)	W/Bern/65 (1)	SL/863/84 (4)	SL/878/84 (1)	SL/10849/84 (1)	SL/10865/84 (1)	W/18537/62 (1)	Mass/9320/77 (1)	SL/853/84 (1)	SL/869/84 (1)	W/171/85 (1)	1973 (1)	A	B
A	1	1436C	2,560	5,120	2,560	3,200	1,280	5,120	5,120	5,120	5,120	5,120	5,120	2,560	5,120	<20	2,560	100	80
		1153	2,560	5,120	640	520	5,120	2,560	5,120	2,560	2,560	640	2,560	640	1,280	<20	1,280	100	80
		1142	2,560	3,840	1,280	600	5,120	1,280	2,560	1,280	640	2,560	2,560	320	2,560	<20	640	100	80
	2a	151	2,560	5,120	2,560	2,400	2,560	5,120	5,120	5,120	5,120	5,120	5,120	5,120	5,120	<20	320	100	80
	2b	1200	2,560	5,120	2,560	1,500	5,120	5,120	5,120	5,120	5,120	640	160	80	160	<20	<20	100	60
	2c	1214	2,560	5,120	2,560	1,700	5,120	5,120	2,560	5,120	2,560	80	40	20	80	<20	<20	100	0
	3	1237	2,560	1,280	640	700	5,120	2,560	2,560	510	960	40	20	40	80	<20	<20	100	0
	4	1129	2,560	2,560	1,280	650	2,560	2,560	2,560	2,560	1,280	1,280	2,560	2,560	2,560	<20	640	100	80
	5	1121	2,560	1,280	640	640	2,560	1,280	2,560	1,280	640	1,280	2,560	2,560	1,280	<20	2,560	100	80
AB	6	1107	2,560	2,560	2,560	1,200	5,120	2,560	5,120	1,280	2,560	5,120	5,120	2,560	5,120	<20	640	100	80
B	7	13-1	<20	80	<20	<20	1,280	320	<20	320	<20	<20	5,120	640	160	160	<20	33	80
	8	43-1	40	2,560	<20	20	2,560	5,120	80	640	80	<20	320	320	160	320	<20	44	80
	9	1112	20	5,120	40	200	5,120	2,560	2,560	20	40	320	5,120	5,120	80	5,120	<20	50	80
	10	1269	160	5,120	2,560	600	5,120	<20	<20	20	<20	<20	<20	<20	320	1,280	<20	61	60
C	11	1243	80	5,120	320	120	5,120	5,120	2,560	320	960	320	5,120	2,560	2,560	640	640	72	100
	12	1331H	1,600	5,120	640	1,300	1,280	5,120	5,120	1,280	1,280	640	5,120	5,120	1,280	5,120	640	100	100
	13	1308F	320	2,560	640	1,050	1,280	1,280	2,560	2,560	640	640	5,120	5,120	2,560	5,120	1,280	100	100
	14	1302A	320	5,120	640	900	2,560	1,280	2,560	640	320	640	5,120	2,560	1,280	2,560	1,280	100	00
		PHS[e]	640	2,560	640	550	320	1,280	2,560	1,280	1,920	2,560	5,120	1,280	1,280	1,280	640	100	100

[a] This table shows the nine reactivity patterns of 18 subgroup A viruses and five reactivity patterns of 5 subgroup B viruses. Each value represents the reciprocal of the neutralization titer of the clinical strain by the MAb. Titers ≤ 80 are considered to be negative and indicate the epitope is absent; they are boxed.

[b] The locations of the regions of isolation for the strains are identified as follows: W, Washington, DC; Aus, Australia; SL, St. Louis; Mass, Massachusetts. The year in which the strain was isolated is given as part of the strain designation. The number of isolates with the indicated reactivity is given in parentheses.

[c] The percentage of clinical isolates neutralized is based on 18 subgroup A and 5 subgroup B isolates. Constant epitopes were present in 100% of the clinical strains examined. For each MAb, the following calculation was done: number of subgroup A (or subgroup B) isolates with a titer >160/total number of subgroup A (or subgroup B) isolates examined. For example, MAb 13-1 neutralized 6 of 18 strains (33%) of the subgroup A isolates examined. Variable epitopes were present on 70 to 99% of the clinical strains, and hypervariable epitopes were present on less than 70% of the strains examined.

[d] The bovine strain of RSV (BRS) was isolated in Iowa.

[e] Human sera that contained high levels of RSV-specific complement-fixing antibody were pooled (PHS) and used to neutralize virus.

TABLE 4. FI and neutralization activities of anti-F MAbs against two subgroup A strains of RSV[a]

Antigenic site	Epitope	MAb	Immunizing strain	Immunoglobulin subclass	Aus/A2/60[b]		SL/863/84[b]		
					N titer	FI titer	N titer	FI titer	N/FI
A	1	1436C	A2	1K	3,200	1,280	5,120	2,560	2
		1153	A2	1K	520	320	2,560	320	8
		1142	A2	1K	600	320	1,280	320	4
	2a	151	A2	1K	2,400	1,280	5,120	2,560	2
	2b	1200	A2	2BK	1,500	1,280	5,120	640	8
	2c	1214	A2	2BK	1,700	640	2,560	640	8
	3	1237	A2	1K	700	640	1,280	320	4
	4	1129	A2	1K	650	320	1,280	320	4
	5	1121	A2	1K	640	640	640	320	2
AB	6	1107	A2	1K	1,200	320	5,120	160	32
B	7	13-1	Long	1K	<20	<20	2,560	<20	>128
	8	43-1	Long	1K	20	<20	5,120	<20	>256
	9	1112	A2	1K	200	<20	5,120	<20	>256
	10	1269	A2	1K	600[c]	320[c]	<20	<20	1.87[c]
C	11	1243	A2	2AK	120	40	5,120	40	128
	12	1331H	A2	2AK	1,300	800	5,120	80	64
	13	1308F	A2	1K	1,050	640	5,120	320	16
	14	1302A	A2	1K	900	640	5,120	64	64

[a] Anti-G cotton rat sera neutralized Aus/A2/60 with N = 2,560 and FI < 20; it neutralized SL/863/84 with FI < 20. Clinical isolate Wash/53/1960 neutralization titers, FI titers, and ratios are as follows: MAb 43-1 (2,560, <20, >256), MAb 13-1 (80, <20, >4), MAb 1112 (5,120, <20, >256), MAb 1269 (5,120, 2,560, 2).

[b] Each value represents the reciprocal of the neutralization (N) titer or FI titer of the isolate by the MAb.

[c] The ratio of neutralization to FI activity for each was determined by using the titer for SL/863/84, except that AUS/A2/60 was used to determine the ratio for MAb 1269.

patterns for the 5 subgroup B viruses (Table 3). The neutralization patterns confirmed the operational map derived from MARM analysis (Table 2) and indicated that MAbs 151, 1200, and 1214 recognized differences in epitopes (Table 3, A2a, A2b, and A2c, respectively). Thus, the total number of neutralization epitopes detectable by the MAb panel is 16. The distribution of neutralization epitopes within strains indicated that constant, variable, and hypervariable regions existed on the F glycoprotein.

Of the detectable F neutralization epitopes, three within site C (C12, C13, and C14) were strictly conserved among all RSV strains examined. In addition, there appears to be a high degree of antigenic stability in site A and AB epitopes among subgroup A viruses: no alterations in site A epitopes were detected. On the other hand, subgroup B viruses showed considerable antigenic variation in site A epitopes: epitopes A1, A2a, A2b, A4, and A5 were unaltered in only 60 to 80% of the subtype B strains analyzed (Table 3, footnote *c*). Epitopes A2c and A3 were not detectable by neutralization of any of the B strains, indicating that these epitopes were subgroup A-specific neutralization epitopes. One subgroup B clinical isolate (W/171/1985) was not neutralized by any of the MAbs reacting with site A or AB epitopes.

In contrast to the antigenic stability in site A and site C epitopes among subgroup A viruses, site B appears to be hypervariable among subgroup A strains. For example, MAbs to site B epitopes neutralized only 33 to 61% of the subgroup A viruses. In contrast, the site B epitopes were relatively conserved among subgroup B viruses (Table 3, footnote *c*).

One bovine RSV isolate was evaluated in a similar manner. This bovine strain, like subgroup B viruses, lacked site A epitopes identified by MAbs 1200, 1214, and 1237. In addition, it had alterations in all four epitopes associated with antigenic site B. This indicates that at least two immundominant regions of the F glycoprotein of bovine RSV are distinctly different from the corresponding regions of human RSV strains.

The results of this analysis indicated that antigenic variation occurs both between and within the human RSV subgroups. However, of the 16 neutralization epitopes identified, 11 were present on each subgroup A virus tested, indicating the high degree of conservation of these F epitopes within type A strains. Subgroup- A-specific neutralization determinants were found in antigenic site A, and hypervariable epitopes clustered in antigenic site B. There was no correlation between the reactivity patterns of the clinical isolates and their year of isolation, indicating that the antigenic variation detected does not represent the progressive accumulation of antigenic alterations with time. Rather, the observed variation appears to be due to genetic heterogeneity among RSV strains that infect humans.

Biological activity of RSV anti-F-MAbs. (i) Fusion inhibition. Previous analysis of other paramyxovirus glycoproteins indicated that distinct functional activities can often be assigned to different antigenic regions of the glycoprotein, e.g., Newcastle disease virus, HN (23); Sendai virus, HN (38); parainfluenza virus type 3, HN, (11); and Newcastle disease virus, F (1). A similar analysis of the antigenic and functional organization of the RSV fusion glycoprotein was performed by comparing the ability of the MAbs to neutralize infectivity and prevent syncytium formation. Expression of neutralization and FI titers (Table 4) as a ratio (N/FI) was used to compare the efficiency of each MAb in neutralization and/or FI and differentiate functional domains on the molecule. Since several site B MAbs exhibited low neutralizing activity against the A2 strain, the assays were also performed with a subgroup A virus (SL/863/84), which was neutralized to high titer by 17 of 18 MAbs (Table 3). These MAbs had similar titers of neutralizing activity but differed substantially in their capacity to prevent the formation of syncytia (Table 4).

TABLE 5. Comparison of neutralizing and binding activities of anti-F MAbs to RSV clinical isolates

Antigenic site	Epitope	MAb	Neutralizing/binding activity of indicated clinical RSV strain[a]				
			AUS/A2/61	W/18537/62	W/3327/66	W/3199/66	W/343/67
A	2C	1214	+/+	−/−	+/+	+/+	+/+
AB	6	1107	+/+	+/+	+/+	+/+	+/+
B	9	1112	+/+	+/+	−/−	−/+	−/+
	10	1269	+/+	−/+	+/+	+/+	+/+
C	11	1243	+/+	+/+	−/−	−/+	+/+

[a] Neutralizing MAbs were tested with the indicated clinical strains in ELISA binding and neutralization tests, and the results of both tests are reported.

Each of the MAbs defining site A epitopes and one MAb defining a site B epitope (B10) inhibited fusion very efficiently, as indicated by the low N/FI titer ratios (ratios of 2 to 8). The majority of site B MAbs did not exhibit FI activity. MAbs to epitopes in site C had intermediate ratios, suggesting that perturbation of this site can also affect fusion function. Thus, it appears that two of the topographically distinct antigenic domains of the F protein correspond to regions that are at or near the site of most important biological activity. Furthermore antigenic sites A and C are relatively well conserved (Table 3), whereas site B is more variable and contains only one epitope that might be at or near the fusion site. Also, it should be emphasized that 6 of the 10 MARMs selected with site A and site AB MAbs had the small-plaque phenotype.

(ii) Neutralization and binding of RSV Anti-F MAbs. The capacity of the RSV F-MAbs to neutralize subgroup A and subgroup B viruses and to bind to the F proteins of these viruses was compared (Table 5). In several instances, MAbs which did not neutralize a given virus also did not bind to the F protein of that particular strain. For example, the lack of binding of MAb 1214 with the 18537 virus explained the failure of this MAb to neutralize this strain. On the other hand, antibody binding did not always correlate directly with the ability to neutralize infectivity. For example, analysis of five other subgroup A clinical isolates yielded additional examples of adequate antibody binding without neutralization. The reactivity of MAb 1112 with isolates W/3199/66 and W/343/67, MAb 1269 with W/18537/62, and MAb 1243 with W/3199/66 illustrate this pattern (Table 5). These results indicate that dissociation of immunological and biological activity can occur for F epitopes and probably reflects the more stringent requirements for antibody-mediated neutralization compared with antibody binding.

DISCUSSION

The goal of this project was to construct detailed operational, topological, and functional maps of neutralization and fusion epitopes on the RSV F glycoprotein. Since immunization of mice with purified RSV proteins appeared to yield few neutralizing MAbs, we devised a novel immunization schedule involving sequential infection with RSV and Vac-F recombinants. This immunization stimulated high levels of F antibody in these animals and resulted in 11 hybridomas which secreted F-specific neutralizing MAbs. The success of this protocol may be due to both the nature of antigen presentation and the degree of stimulus obtained by infecting mice with two different viruses in a specific order. First, immunization by virus infection allowed the F glycoprotein to be delivered in an undenatured form. Second, mucosal infection with RSV primed the animal for an F response but did not restrict the replication of Vac-F. Priming by natural infection with RSV also appeared to be important. Anti-F titers obtained after immunization with Vac-F alone or induced by infection with Vac-F followed by RSV were much lower than we have reported here (data not shown).

Competitive radioimmunoassay with these MAbs identified three nonoverlapping antigenic sites involved in neutralization. We also observed a potential bridge site (AB), suggesting that sites A and B are in close proximity on the F molecule. These results are in good agreement with those obtained for RSV and other paramyxovirus fusion proteins, which have delineated three or four nonoverlapping antigenic regions (4, 10, 14, 15, 42, 50). Furthermore, neutralization tests involving MARMs and RSV clinical isolates proved to be the most sensitive method to detect subtle antigenic differences among these viruses; they identified 16 epitopes. Seven of these epitopes are located within site A, four are located within site B, four are located within site C, and one is located in the bridge site AB. One of the neutralization epitopes of the RSV F-glycoprotein has already been localized to residues 221 to 236 on the F_1 subunit by Trudel et al. (48). Nucleotide sequence analysis of our MARMs will give additional information about the specific amino acids involved in 13 of the neutralization epitopes of the RSV F glycoprotein.

This study also yielded additional insights into the behavior of F neutralization epitopes in response to mutations within specific antigenic sites. We present evidence that mutations distant from the antigenic site can effect resistance to neutralization or enhancement of neutralization, presumably mediated by conformational changes. MARMs with mutations that abolish reactivity with site A MAbs also increased the reactivity of certain site B epitopes or abolished neutralization mediated by a site C epitope. One MARM, V1107, sustained a mutation in the bridge epitope AB, and, as a result, neutralization at a distant epitope, C14, was abolished. Similarly, one site C MARM, V1302A, failed to be neutralized by the MAb directed against antigenic site A epitope 3 and was also neutralized by site B MAbs 13-1 and 43-1 at titers of 1/1,280 and 1/5,120, respectively. These results indicate that local changes in the F glycoprotein can result in profound changes in the structure of epitopes located on more distant antigenic regions.

This is also the first report of MAb-selected small-plaque variants of RSV. These six viruses implicate four site A epitopes and the bridge site, AB, as important functional regions on the F glycoprotein that may regulate the fusion of infected cells. Specific mutations within the F glycoprotein of other paramyxoviruses have been associated with alterations in plaque size. Indeed, a MAb-selected small-plaque variant of Sendai virus has an amino acid change in Sendai virus F1 residue 407 (Pro→Gln), and it has been proposed that this epitope is part of the fusion-active site (39). Protease-resistant mutants of Sendai virus are another example of small-plaque variants. It has been determined that the

alteration in the biological properties of these viruses occurs as a result of specific amino acid changes at or near the cleavage site (22, 24). It is anticipated that our small-plaque-phenotype RSV MARMs will likewise have specific amino acid changes in their F glycoproteins. It is unlikely, although still possible, that these phenotypic changes are the result of modifications in other envelope glycoproteins or the matrix protein as described for mumps virus, measles virus, and bovine parainfluenza virus type 3 (7, 29, 43). The mechanism by which these mutations affect plaque size remains to be studied. One can postulate several causes, including a quantitative decrease in the amount of F glycoprotein expressed at the surface of infected cells. The overall stability of the F glycoprotein may be altered as a result of changes in charge or hydrophobicity, and this variant F molecule might be less likely to interact with adjacent cell membranes or have decreased activity during syncytium formation.

We were able to delineate antigenic sites and epitopes involved in syncytium formation by fusion inhibition assays. Thus, two of the antigenically stable neutralization sites on the F glycoprotein (sites A and C) correspond to protein domains important for viral fusion activity. A third neutralization site (site B) has minimal involvement in syncytium formation, which may account for the high degree of variation tolerated in this domain. The fact that site A MAbs inhibit syncytium formation more efficiently than site C MAbs and most site B MAbs do has no effect on syncytium formation may reflect differences in the proximity of these three antigenic sites to the fusion-active site(s). Thus, antigenic site A may be at or near a fusion-active site, because it appears to have an important effect on plaque size and is involved in syncytium formation. In addition, we have been able to characterize both MARMs and a clinical isolate which escaped neutralization by all of the MAbs directed to site A or site C epitopes. These viruses form syncytia in vitro, suggesting that major antigenic changes in the F glycoprotein can be tolerated without loss of the fusion-active site.

Another interesting structural property of F-glycoprotein epitopes became apparent when we compared the reactivity patterns of MAbs in neutralization and binding assays. We discovered that there is not necessarily a direct correlation between these two activities. As was expected, absence of reactivity in ELISA did indicate that the epitope for neutralization was absent; however, there were several cases, particularly for site B epitopes, that demonstrated dissociation in biological and immunological reactivities, i.e., MAbs could bind but no longer neutralize. Similar findings have been noted in the course of differentiating strains of Sindbis virus (45), yellow fever virus (6), rabies virus (17), and human parainfluenza virus type 3 (10). Currently, we lack a satisfactory explanation for this common but puzzling observation. It may imply that sequence changes in these epitopes result in escape from neutralization but that the overall conformation has not deviated from the wild type sufficiently to preclude binding. This may also be an indication that major conformational changes in these epitopes would not be tolerated, because they contribute to the stability of biologically functional regions. Dramatic changes would produce lethal mutations and may explain, in part, our failure to select MARMs in three of four site B epitopes despite repeated attempts. Furthermore, it also indicates that the use of only binding assays may lead to an underestimate of the true epidemiological potential of certain RSV strains.

Thus, the combination of MARMs and biological assays allowed us not only to map F neutralization epitopes more thoroughly, but also to characterize important structural and functional relationships. Comparison of the F gene nucleotide sequences of the RSV MARMs described here with those of human parainfluenza type 3 fusion protein MARMs (10) will identify specific areas of paramyxovirus F glycoproteins critical for neutralization and fusion inhibition by monoclonal antibodies.

Cross-neutralization studies were also used to evaluate the antigenic variation in these F epitopes that occurs in nature. RSV strain variation was initially observed by Coates et al. (9) and has been confirmed in more recent epidemiological studies by Mufson et al. (31) and Hendry et al. (21), using binding assays. We were able to detect antigenic variation in the F epitopes of RSV clinical strains and to identify constant, variable, and hypervariable regions of the molecule (Table 3, footnote *c*) as well as subgroup-specific neutralization epitopes. Of 11 epitopes of antigenic sites A and C, 10 were constant among subgroup A viruses; however, several epitopes in site A were highly variable among subgroup B viruses. In contrast to the generally conserved nature of sites A and C, site B varies among both subgroup A and B strains and appears to be hypervariable among subgroup A isolates. Successful vaccine design should include, as a minimum, the epitopes of antigenic site C to stimulate neutralizing antibody that will cross-react with all subgroup A and B viruses.

Although the clinical relevance of RSV F-epitope variation is unknown, it does raise interesting questions regarding the immune response to natural infection and immunization. Which F epitopes should be present in a vaccine to stimulate complete and durable protection against disease in vivo? Is there a desirable repertoire of responses that will prevent the spread of infection? Are antibody responses to specific epitopes antagonistic or detrimental in vivo? Wang et al. have indicated that such restrictions do occur in individual antibody responses in their investigation of anti-influenza hemagglutinin epitope-specific responses following natural infection (51). They have observed that individuals may respond differently to infection by producing antibodies with a wide range of specificities and thereby demonstrated that no single region of the hemagglutinin molecule was immunodominant. A similar situation may exist for other viral envelope glycoproteins, including RSV.

The spectrum of the immune response of infants and children to the neutralization epitopes of the RSV F glycoprotein following initial infection is unknown at present. If these epitopes are not equally immunogenic during infection of humans, we can postulate, on the basis of the results or the present study, that primary exposure to a subgroup A virus with alterations in all site B epitopes might not confer adequate protection in vivo against subsequent infection with a subgroup B virus which has the commonly conserved site B epitopes but not the conserved epitopes of antigenic sites A and AB. Thus, individual epitope-specific responses may explain, in part, the reinfection of seropositive children such as those described in the study by Mufson et al. (31). What is needed is a study that would combine the evaluation of the specific response of infants and children to the neutralization epitopes on the RSV F glycoprotein with F-epitope analysis of the RSV strains shed from each child during the initial and any subsequent infection. This would allow the identification of F epitopes that are immunodominant during human infection and would give additional

insight into the development of a protective humoral immune response.

This study has provided a detailed characterization of the neutralization and fusion epitopes on the F glycoprotein of RSV. In addition, we have defined important structure-function relationships that suggest that neutralization epitopes occupy operationally interdependent domains on an extremely flexible molecule. We are currently sequencing the F genes of our MARMs to identify the amino acids involved in specific epitopes, especially those that are critical to fusion activity.

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